Biochimica et Biophysica Acta 1798 (2010) 291–296

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Evidence of phosphatidylethanolamine and phosphatidylglycerol presence at the annular region of lactose permease of *Escherichia coli*

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ARTICLE INFO

Article history:
Received 17 March 2009
Received in revised form 15 June 2009
Accepted 29 June 2009
Available online 10 July 2009

Keywords:
Annular lipid
Lactose permease
Lipid–protein interaction
Förster resonance energy transfer

ABSTRACT

Biochemical and structural work has revealed the importance of phospholipids in biogenesis, folding and functional modulation of membrane proteins. Therefore, the nature of protein–phospholipid interaction is critical to understand such processes. Here, we have studied the interaction of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) mixtures with the lactose permease (LacY), the sugar/H^+ symporter from *Escherichia coli* and a well characterized membrane transport protein. FRET measurements between single-W151/C154G LacY reconstituted in a lipid mixture composed of POPE and POPG at different molar ratios and pyrene-labeled PE or PG revealed a different phospholipid distribution between the annular region of LacY and the bulk lipid phase. Results also showed that both PE and PG can be part of the annular region, being PE the predominant when the PE:PG molar ratio mimics the membrane of *E. coli*. Furthermore, changes in the thermotropic behavior of phospholipids located in this annular region confirm that the interaction between LacY and PE is stronger than that of LacY and PG. Since PE is a proton donor, the results obtained here are discussed in the context of the transport mechanism of LacY.

1. Introduction

Membrane transport proteins utilize the Gibbs energy stored in the electrochemical ion gradient ($\Delta\tilde{\mu}_i$)[2] to drive the uphill translocation of substrates across the cell membrane [1]. The marked increase in the number of known 3D structures of this type of membrane proteins over the last 6 years clearly indicates that most research in the transport field is focused on the molecular mechanism by which these proteins transduce the Gibbs energy from ion gradients [2]. In contrast, less attention has been paid to the role of the lipid bilayers in the origin and maintenance of these gradients. Clearly, the possible direct role of membrane phospholipids in such processes [3] and how phospholipids modulate the function of membrane transport proteins is still far from being understood.

Recent work in crystallography has increased interest in the role of phospholipids in maintaining the structural architecture of membrane proteins. For instance, several crystal structures have revealed the presence of phospholipids attached to the lipid-exposed face of the protein [4,5]. Notably, the addition of specific phospholipids to the detergent-purified protein has been the key to obtaining suitable crystals for X-ray diffraction [5,6]. Besides, experimental evidence has shown the importance of phospholipids in protein stability and activity. For example, van Dalen demonstrated the role of phosphatidylethanolamine (PE) in the efficient membrane assembly of the four oligomers that form the functional unit of the potassium channel, KcsA [7].

It is widely believed that all integral membrane proteins are surrounded by a layer of phospholipids, known as annular or boundary lipids, whose composition and physical properties may play a role in protein function [8]. In physical terms, the boundary region should be thick enough to embed the protein; in other words, the length of the hydrophobic domain of the protein has to match the thickness of the lipid bilayer [9]. For this reason (hydrophobic matching) it is assumed that the phospholipid species that form the boundary or annular region are selective for a particular protein and a particular function in the membrane [10].

One of the best paradigms of secondary membrane transporters is the lactose permease of *Escherichia coli* (LacY) [11]. LacY is a polytopic membrane protein (12 transmembrane regions) that catalyzes the coupled stoichiometric translocation of a galactoside

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[2] In the bioenergetic field, for the electrochemical gradient of H^+ the "proton-motive force" is often used, defined as: $\Delta p = \Delta\tilde{\mu}_{H^+} / F$.

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doi:10.1016/j.bbamem.2009.06.024

with an H^+ across the membrane. Like KcsA, it was earlier demonstrated that LacY requires the presence of PE for both its correct folding in the membrane during biogenesis [12] and its function *in vivo* [13]. More recently, it was observed in cells lacking PE that the N-terminal six transmembrane bundle was inverted with respect to the plane of the membrane bilayer, maintaining the correct topology of the C-terminal helical bundle [14,15]. For all these reasons, since LacY is fully functional when reconstituted in mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) it is believed that these phospholipids obey the matching principle and that, one or both, may form part of the annular region. Accordingly, the main goal of this study is to examine the selectivity of LacY for POPE and/or POPG.

Besides electron spin resonance (ESR) [16], Förster resonance energy transfer (FRET) has been successfully used in the investigation of membrane protein–lipid selectivity [17] and appears to be a suitable technique to examine the boundary region of LacY. The FRET strategy, in the framework of this study, consists of measuring the efficiency of the energy transfer between W151 of LacY used as a donor (D), and different pyrene-labeled phospholipids as acceptors (A). In particular, two phospholipid analogues of PG and PE labeled with pyrene, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Pyr-PG) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine ammonium salt (Pyr-PE), were used as acceptors.

In summary, by exploiting pyrene fluorescence emission properties and FRET tools we have examined the selectivity of LacY annular region for either POPE or POPG. In addition, phospholipids are thermotropic and show well-defined phase transition behavior from the gel (L_β) to liquid-crystalline (L_α) phases. Therefore to explore the protein–phospholipid interaction we have studied the dependence of the LacY-POPE/POPG interaction with respect to temperature.

2. Materials and methods

2.1. Materials

N-dodecyl-β-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine (POPE), and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Pyr-PG) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine ammonium salt (Pyr-PE) were purchased from Invitrogen (Barcelona, Spain). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Bio-Beads SM-2 was purchased from Bio-Rad (Hercules, CA, USA). All other common chemicals were ACS grade.

2.2. Bacterial strains and protein purification

Plasmid pCS19 encoding single-W151/C154G LacY with a 6-His tag at the C terminus was generated as described [18] and provided by Dr. H. Ronald Kaback (UCLA, USA). *E. coli* BL21(DE3) cells (Novagen, Madison, WI, USA) transformed with this plasmid were grown in 6.4 l of Luria-Bertani broth at 30 °C containing ampicillin (100 μg/ml) to an absorbance (600 nm) of 0.6 and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were disrupted by passage through a French pressure cell, and the membrane fraction was harvested by ultracentrifugation. Membranes were solubilized by adding DDM to a final concentration of 2%, and LacY was purified by Co (II) affinity chromatography (Talon Superflow™, Palo Alto, CA, USA). Protein eluted with 150 mM imidazole was dialyzed against 20 mM Tris–HCl (pH 7.5), 0.008% DDM, concentrated by using Vivaspin 20 concentrators (30 kDa cutoff; Vivascience, Germany) and stored on ice. As determined by sodium dodecylsulfate/12% polyacrylamide gel

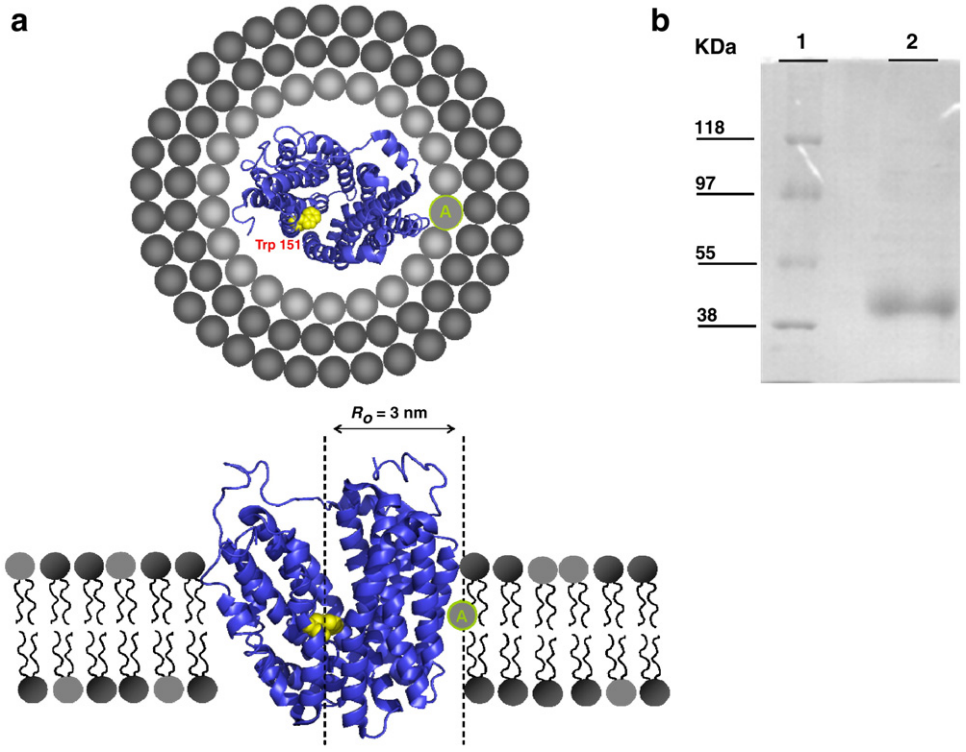

Fig. 1. Model of lactose permease embedded in the bilayer: sagittal view showing the first annular shell (top); and frontal view showing the location of the W151 residue (donor) and the acceptor (A) (Pyr-PE or Pyr-PG) (bottom) (a). Molecular weight marker (1) and LacY single-W151 (2) band intensities obtained by direct staining during Coomassie blue–SDS–PAGE (b).

electrophoresis followed by Coomassie blue staining, the preparations contained only a single band with an apparent molecular weight of 36 kDa. Protein was assayed by using a micro-BCA kit (Pierce, Rockford, IL).

2.3. Vesicle preparation and protein reconstitution

Liposomes and proteoliposomes were prepared according to methods elsewhere published [19]. Briefly, chloroform:methanol (3:1, v/v) solutions containing appropriate amounts of POPE, POPG and PyrPG or PyrPE were dried under a stream of oxygen-free N_2 in a conical tube. The total concentration of phospholipids was 100 μM. The resulting thin film was kept under high vacuum for approximately 3 h to remove organic solvent traces. Multilamellar liposomes (MLVs) were obtained following redispersion of the film in 20 mM Hepes, 150 mM NaCl buffer, pH 7.40, and application successive cycles of freezing and thawing below and above the phase transition of the phospholipids, and sonication for 2 min in a bath sonicator. Afterwards, large unilamellar liposomes (LUVs) supplemented with 0.2% of DDM were incubated overnight at room temperature. Liposomes were subsequently mixed with the solubilized protein and incubated at 4 °C for 30 min with gentle agitation, to obtain a lipid-to-protein ratio (LPR) (w/w) of 40. DDM was extracted by addition of polystyrene beads (Bio-Beads SM-2, Bio-Rad) as described elsewhere [20].

2.4. Fluorescence measurements

Steady state fluorescence measurements were carried out with an SLM-Aminco 8100 (Urbana, IL, USA) spectrofluorometer. The cuvette holder was thermostated with a circulating bath. The fluorescence was recorded at two degree intervals in the range of 5 to 30 °C. The temperature was controlled within 0.1 °C using a circulating water bath (Haake, Germany). The excitation and emission bandwidths were 4/4 and 8/8 nm, respectively. Annular and bulk fluidity were determined as described elsewhere [21]. Pyrene fluorescence was excited at 338 nm, with fluorescence spectra scanned from 350 to 500 nm. For energy transfer measurements, Trp was excited at 295 nm, and the spectra recorded from 300 to 500 nm. To calculate the excimer to monomer fluorescence ratio (E/M), we used signal intensities at 375 nm (corresponding to the peak of monomer band) and 470 nm (maximum of pyrene excimer band). Based on the quenching of intrinsic tryptophan by pyrene phospholipids, the values of experimental energy transfer efficiency (E) were determined according to the equation

$$E = 1 - \frac{I_{DA}}{I_D} \quad (1)$$

where I_D and I_{DA} are the tryptophan emission intensities in the absence or presence of pyrene phospholipid derivative, respectively [22]. The I_D intensities were evaluated from the peak height of the 338 nm tryptophan fluorescence [23] under excitation at 295 nm and the spectra were recorded from 300 to 400 nm. Quenching data were corrected by following the procedure described elsewhere [24].

Distance R is derived from

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (2)$$

where R_0 is the Förster radius [22] and E the energy transfer efficiency.

2.5. Differential scanning calorimetry

Large unilamellar vesicles (LUVs) to be used for DSC studies were prepared as described above to a final concentration of 2.74 mM. DSC analyses were performed using a MicroCal MC-2 calorimeter following procedures described elsewhere [25]. Data were analyzed using the original calorimeter software. T_m was taken as the temperature of maximum excess specific heat (and measured to the nearest 0.5 °C). Transition enthalpy was calculated as described elsewhere [26]. The calorimetry accuracy for T_m and for enthalpy changes was ±0.1 °C and ±0.2 kcal mol^{-1} respectively. Each sample was scanned in triplicate over the temperature range 5–40 °C at a scan rate of 0.44 °C·min^{-1}.

3. Results and discussion

To perform the FRET experiments, we used a single tryptophan mutant of LacY, the single-W151 LacY with an extra mutation: C154G (Fig. 1a). This mutant was generated after replacing five of the six natural tryptophans (Trp10, Trp33, Trp78, Trp171 and Trp 223) by tyrosine and mutating Cys154 by glycine [18]. As in the wild-type [27], the C154G mutation in the single-W151 background of LacY increases the stability of the protein in detergent-purified solution. This mutant recognizes the substrate to a similar extent as the wild-type but is unable to translocate it [28]. Both crystal structures of the C154G-LacY [29] and the wild-type LacY [30] show that W151 is part of the substrate binding site and is located in a relatively hydrophilic environment near the geometrical centre of the protein equidistant from either side of the membrane (Fig. 1a).

The fluorescence spectra of Pyr-PE or Pyr-PG display the two characteristic peaks (Fig. 2a) described in the literature [22]: the 375 nm band, typical of the monomer (M), and the 470 nm band, characteristic of the excimer (E). Since the excimers are formed when an excited pyrene molecule interacts with a neighboring unexcited

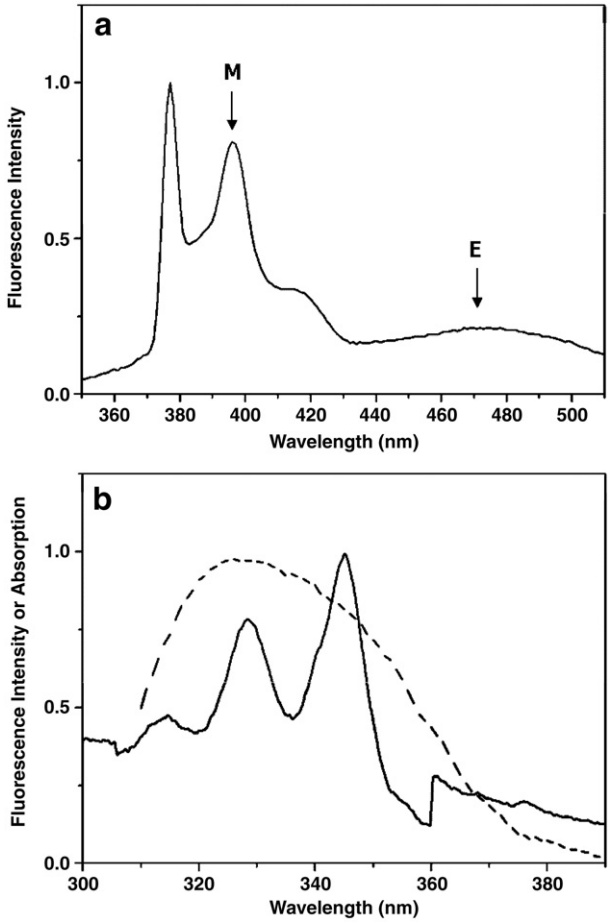

Fig. 2. Fluorescence spectra of Pyr-PG showing the monomer (375 nm) and excimer (470 nm) bands (a). Overlap between the emission spectrum of the LacY mutant single-W151/G154C (dashed line) and the excitation spectra of proteoliposomes bearing the pyrene derivative (solid line) (b). Spectra have been peak normalized for protein concentration.

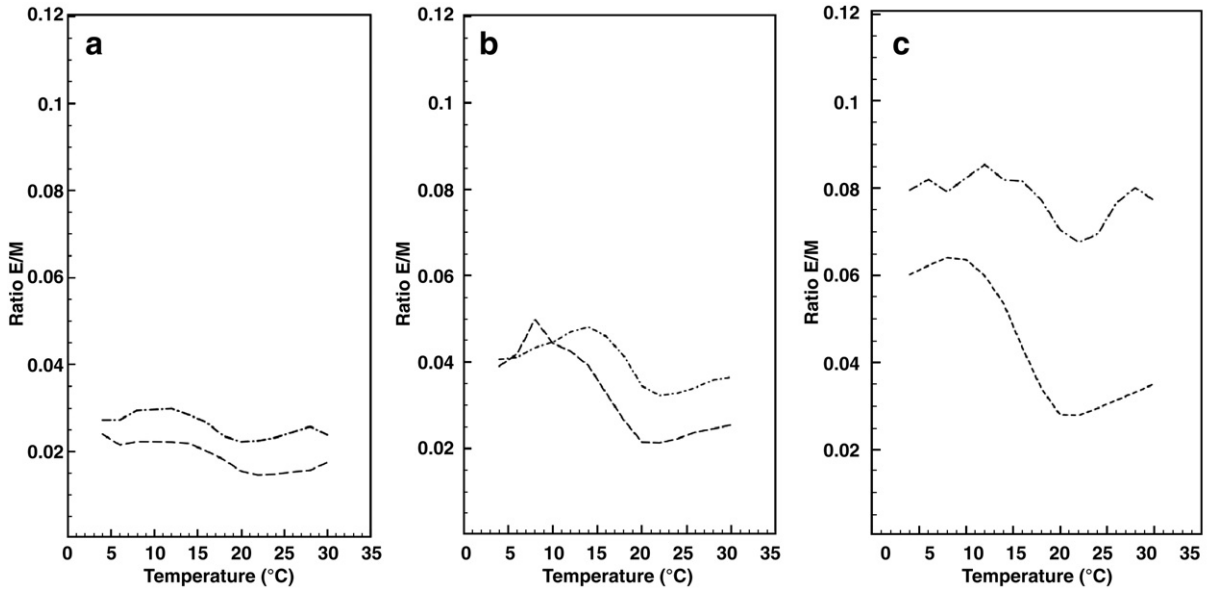

Fig. 3. Temperature dependence of the excimer to monomer ratio (E/M) of liposomes (a) and proteoliposomes of POPE/POPG labeled with Pyr-PG (dashed line) and Pyr-PE (dashed-dotted line) when excited at its own wavelength (bulk region) (b); and by W151/G154C (annular region) (c).

pyrene during its fluorescence lifetime, the specific E/M ratio normally provides information about the lateral diffusion mobility of the labeled phospholipids. Furthermore, when the pyrene-labeled phospholipids are directly excited at their own wavelength (338 nm) the E/M ratios provide information on the lateral diffusion of the labeled phospholipids of the non-annular sites, namely, the global or bulk fluidity. On the other hand since the emission spectrum of W151 and the excitation spectrum of pyrene phospholipid derivatives (Pyr-PE or Pyr-PG) as acceptors overlap (Fig. 2b), we can examine phospholipid proximity for LacY using these donor–acceptor pairs by resonance energy transfer spectroscopy. The theoretical distance between W151 and the acceptor molecules in the annular region of the protein (Fig. 1) is within the measurement range of FRET when using these donor–acceptor pairs (R_0 ~3 nm) [31]. Therefore, we can selectively excite the pyrene-labeled phospholipids located in the immediate vicinity of the protein by indirectly exciting them through W151 (excitation at 295 nm) via a FRET mechanism. Consequently the E/M ratio observed in this last situation will provide information on the selectivity of a particular phospholipid for the annular region of the protein. In addition, phospholipids are thermotropic and show well-defined phase transition behavior from L_β to L_α phases. Therefore to explore the protein–phospholipid interaction we also examined the dependence of the E/M ratios on temperature.

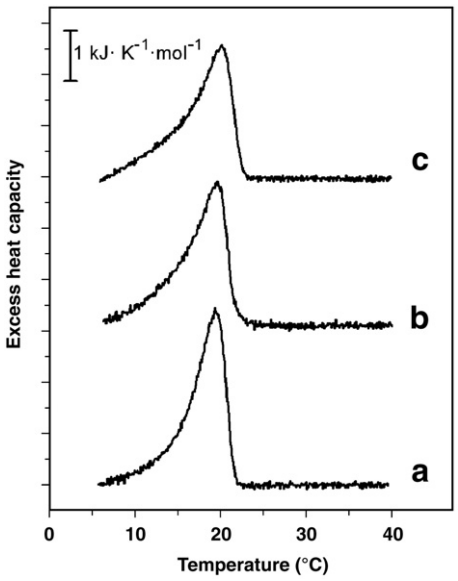

Fig. 4. Excess heat capacity measured as a function of temperature for POPE/POPG (75:25, mol/mol) (a), POPE/POPG/Pyr-PE (72:25:3, mol/mol/mol) (b) and POPE/POPG/Pyr-PG (75:22:3, mol/mol/mol) (c), LUVs (total phospholipid concentration: 2.74 mM).

The experiments were performed after reconstituting the purified protein into bilayers formed by the most abundant phospholipids in the inner membrane of *E. coli* composed roughly of ~70% PE, 25% PG and 5% of other phospholipid species [32]. Single-W151/C154G LacY was reconstituted in two different systems: POPE/POPG/Pyr-PG (75:22:3, mol/mol/mol) and POPE/POPG/Pyr-PE (72:25:3, mol/mol/mol). As can be seen in the plots of the E/M ratios for liposomes (Fig. 3a) and proteoliposomes, global and annular regions (Fig. 3b and c, respectively), of both systems consist of three regions: an initial increase with increasing temperature followed by a more or less abrupt decrease and, finally, an increase, again, with temperature. This behavior, sometimes referred to as N-shaped curves [33], reflects the L_β to L_α phase transition of the phospholipid matrix and was earlier reported in the phospholipids of reference such as DPPC [34–36].

In order to illustrate the partition behavior of the pyrene lipids in our phospholipid binary mixture, Fig. 4 shows the normalized DSC endotherms of POPE/POPG (75:25, mol/mol) in the absence/presence of the pyrene-labeled POPE or POPG. No significant differences were observed between the three consecutive scans obtained for each sample. Table 1 also lists the values of the thermodynamic parameters of the phase transitions observed: the temperature of maximal excess

Table 1
Thermodynamic parameters of the phase transitions of POPE:POPG (75:25) mixtures in the absence/presence of pyrene probe.

Sample	T_m (°C)	ΔT (°C)	ΔH (kJ·mol^{-1})
POPE/POPG (75:25, mol/mol)	19.3	4.1	17.2
POPE:POPG:Pyr-PE (72:25:3, mol/mol/mol)	19.6	5.2	17.7
POPE:POPG:Pyr-PG (72:25:3, mol/mol/mol)	19.9	5.3	16.9

Table 2
Distances (R) between W151 and Pyr-PG and Pyr-PE at 10 °C and 26 °C calculated from Eq. (2).

Temperature (°C)	Lipid proportion POPE:POPG (mol/mol)	Distance, R (nm)	
		Pyr-PE	Pyr-PG
10	25/75		3.74
	50/50	3.83	3.27
	75/25	2.77	2.93
26	25/75		4.41
	50/50	4.01	3.83
	75/25	2.69	2.83

heat capacity (T_m,°C), the peak width at half height (ΔT, °C) and the transition enthalpy change (ΔH, kJ·mol^{-1}).

The heat capacity curve in the absence of a fluorescent probe (Fig. 4, trace a) is slightly asymmetric, skewed to the low temperature side, indicating non-ideal mixing behavior characteristic of a mixture of phospholipids with the same chains but different head groups [37]. The presence of Pyr-PE or Pyr-PG (traces b and c) causes the endotherm to widen slightly (see Table 1) indicating enhanced deviation from ideal behavior without phase segregation. A non-ideal mixing of pyrene probes with phospholipid bilayers without phase separation has been previously described in DPPC [34].

Since for POPE:POPG liposomes the T_m has been established by DSC in ~20.0°C, the E/M ratios versus T in Fig. 3 reflect the L_β to L_α phase transition of this mixture. The discrepancy between the T_m value obtained by DSC and the lower values that can be estimated from the midpoint transition of the N-shaped plots in Fig. 3 may be attributed to the inherent differences between the two techniques, probably implying a higher accuracy for the DSC outcome.

The E/M ratios for Pyr-PE and Pyr-PG liposomes (Fig. 3a) were similar in shape but the values were lower than those calculated for the global (Fig. 3b) and annular (Fig. 3c) regions. In addition, the L_β to L_α phase transition becomes sharper and more pronounced in the presence of the protein. Although we have no explanation at the molecular level, these observations may result from the interaction between the protein and the phospholipids. Notably, in all range of temperatures, E/M values for Pyr-PE were always higher than those for Pyr-PG. Since, in general terms, E/M ratios reflect the fluidity of the bilayers [38], these results would indicate that Pyr-PE molecules are in more fluid environment than Pyr-PG molecules. However, these differences were lower in both the liposomes (Fig. 3a) and in the global region (Fig. 3b) than in the annular region (Fig. 3c). These results suggest a different distribution of the labeled phospholipids between the global and the annular region due to the presence of the protein. That is, the presence of LacY induces a redistribution of phospholipids.

A straight interpretation of E/M ratios as a direct measure of the fluidity is not satisfactory when pyrene molecules are confined [39], as occurs in the annular region. More likely, our observations suggest that PE (assuming that it behaves as Pyr-PE) could be more predominant than PG at the annular region of LacY. Hence, the probability of excimer formation within this confined region will be higher for Pyr-PE than for Pyr-PG.

Apparently, our observations would be in contradiction with electron spin resonance experiments that show that lipids are more immobilized near the protein [16]. Hence in such situations the fluidity (E/M ratios) reported by the annular phospholipids is expected to be lower than the global. However, the term immobilized means that the phospholipids are not as well oriented as the global phospholipids. Indeed to match the irregular surface of the protein, the lipid acyl chains become distorted, leading to an annular shell covered by disordered and motionally restricted phospholipids [10]. In such a case, the number of Pyr-PE excimers at the annular region will always be higher than that in the rest of the bilayer. Therefore, in the presence of the protein the E/M ratio would not reflect the fluidity of the annular region but the high probability of excimer formation. This may explain the high E/M values reported by Pyr-PE for this region (Fig. 3c).

The increase in excimer formation was earlier interpreted as a result of the segregation of pyrene-labeled phospholipids in the vicinity of LacY [40]. In concordance, we now extend this observation to the biomimetic mixture formed by POPE:POPG, where the labeled phospholipids report the predominance of PE at the annular region of LacY. PE is known to act as a lipochaperone for LacY [41], interacting transiently with folding intermediates of the protein. In addition, there is evidence from *in vivo* experiments that PE may also interact with the folded protein [12,14,42]. So far, our results provide means for a possible direct interaction of PE, as a putative proton phospholipid donor, with the protein.

Actually, to ascertain if there is an enrichment in PE at the annular region we have performed FRET experiments between single-W151/C154G LacY and three different POPE:POPG molar compositions containing either Pyr-PG or Pyr-PE. As can be seen in Fig. 5 the highest transference of energy occurs for the mixture POPE:POPG (75:25, mol/mol) which mimetizes the phospholipid molar ratio of the inner membrane of *E. coli* and for which LacY is fully functional. In such case the energy transfer efficiency is small but higher for Pyr-PE than for Pyr-PG. It is interesting, however, to analyze the situation for the other two compositions. Thus, for the POPE:POPG (50:50, mol/mol) mixture we observe that the transference of energy becomes lower than for the POPE:POPG (75:25, mol/mol) composition at both temperatures. Even more remarkable is the fact that the efficiency becomes larger for Pyr-PG than for Pyr-PE. Besides, we observe a more dramatic decrease in the efficiency for the POPE:POPG (25:75, mol/mol) for Pyr-PG and a nonmeasurable efficiency transfer to Pyr-PE. Probably because in this last case the pyrene moiety has been excluded from the annular region and diluted in the bulk. On the other hand at low PE proportions LacY might undergo changes in its native topology leading to a different distribution of the phospholipids around the protein [14,15,43]. These experiments are important as control and evidence a dilution effect of PE when increasing the molar proportion of PG in the mixtures. On the other hand, according to the Förster's theory and by inserting the efficiency values in Eq. (2) we can obtain an estimation of the average distances between the W151 position and the pyrene moiety of the two labeled phospholipids in the annular region (Table 2). Basically, the values of the average R for

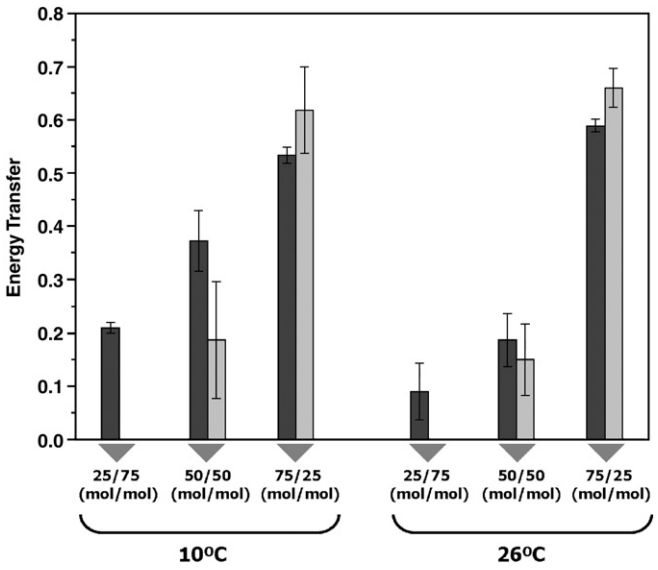

Fig. 5. Efficiency of the energy transfer between W151 and Pyr-PG (black) and Pyr-PE (grey) at 10 °C and 26 °C at different POPE:POPG molar ratios. Error bars show the SDs of the average of three independent experiments.

all compositions, below and above the T_m of the lipid matrix, fall within the range of expected annular distances for LacY. Therefore we can conclude that both phospholipids may form part of the annular region. For the POPE:POPG (75:25, mol/mol) mixture the calculated average *R* values show that PE is slightly closer to W151 than PG (being the difference between them ~0.15 nm). It is worth to mention that this difference is reproducible and might be significant at the nanoscale level. However, since we have one donor and multiple acceptors these values should be interpreted as a consequence of a possible major density of Pyr-PE in the annular region. As can be seen in Fig. 5 the efficiency of energy transfer depends on the lipid composition and the maximum values appear in the biomimetic lipid composition (POPE:POPG, 75:25, mol/mol). This indicates that the optimal matching between the phospholipids in the annular region and LacY occurs at this particular composition. Our results suggest that the annulus is not formed exclusively by PE and that PG is also necessary to functionally reconstitute LacY. This provides mean for the similar values of *R* obtained for PE and PG, respectively. The presence of PG in addition to PE in the annular region of LacY seems to be necessary for a correct hydrophobic mismatching between LacY and the phospholipid phase [9,10]. Finally, since PE is a good proton donor, we hypothesise that PE may act as a bridge or, perhaps, may directly provide to LacY the H^+ necessary to trigger the H^+/sugar coupled translocation [11]. We believe that the functional and structural roles of PE and other phospholipids during active transport mediated by transmembrane proteins need to be further investigated. Current research conducted in our laboratory is continuing in this direction.

Acknowledgements

L.P. is recipient of fellowship APIF of the U.B. This work has been supported by Grant CTQ-2008-03922/BQU from the Ministerio de Ciencia e Innovación of Spain. Alvin Thomas is thanked for technical assistance. Thanks to Dr. Manuel Palacín for the use of his laboratory. The authors specially thank Professor H. Ronald Kaback for providing the LacY expression vector.

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